

AD-A224 109 RT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT TYPE Unclassified		1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY DTIC JUL 19 1980 SELECTED		3. DISTRIBUTION/AVAILABILITY OF REPORT Approval for public release; distribution unlimited	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
4. PERFORMING ORGANIZATION REPORT NUMBER		7a. NAME OF MONITORING ORGANIZATION	
6a. NAME OF PERFORMING ORGANIZATION DCD&I Department of Enteric Infections	6b. OFFICE SYMBOL (if applicable)	7b. ADDRESS (City, State, and ZIP Code)	
6c. ADDRESS (City, State, and ZIP Code)		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (if applicable)	10. SOURCE OF FUNDING NUMBERS	
8c. ADDRESS (City, State, and ZIP Code)		PROGRAM ELEMENT NO. 6110zA	PROJECT NO. 3M16102BS1
		TASK NO. AE	WORK UNIT ACCESSION NO. 207 WGG3
11. TITLE (Include Security Classification) Molecular cloning and sequence analysis of the Sta58 major antigen gene of Rickettsia tsutsugamushi: Sequence homology and antigenic comparison to the 60-kilodalton family of stress proteins.			
12. PERSONAL AUTHOR(S) Charles K. Stover, David P. Marana, Gregory A. Dasch, and Edwin V. Oaks			
13a. TYPE OF REPORT scientific report	13b. TIME COVERED FROM TO	14. DATE OF REPORT (Year, Month, Day) 90/3/09	15. PAGE COUNT 9
16. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
		Rickettsia tsutsugamushi, antigens, molecular cloning, scrub typhus, heat-shock proteins, nucleotide sequence.	
19. ABSTRACT (Continue on reverse if necessary and identify by block number) The scrub typhus 58-kilodalton (kDa) antigen (Sta58) of Rickettsia tsutsugamushi is a major protein antigen often recognized by humans infected with scrub typhus rickettsiae. A 2.9 kilobase HindIII fragment containing a complete sta58 gene was cloned in Escherichia coli and found to express the entire Sta58 antigen and a smaller protein with an apparent molecular mass of 11 kDa (Stp11). DNA sequence analysis of the 2.9-kilobase HindIII fragment revealed two adjacent open reading frames encoding proteins of 11 (Stp11) and 60 kDa (Sta58). Comparisons of deduced amino acid sequences disclosed a high degree of homology between the R. tsutsugamushi proteins Stp11 and Sta58 and the E. coli proteins GroES and GroEL, respectively, and the family of primordial heat shock proteins designated Hsp10Hsp60. Although the sequence homology between the Sta58 antigen and the Hsp60 protein family is striking, the Sta58 protein appeared to be antigenically distinct among a sample of other bacterial Hsp60 homologs, including the typhus group of rickettsiae. The antigenic uniqueness of the Sta58 antigen indicates that this protein may be a potentially protective antigen and a useful diagnostic reagent for scrub typhus fever.			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION	
22a. NAME OF RESPONSIBLE INDIVIDUAL		22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL

Molecular Cloning and Sequence Analysis of the Sta58 Major Antigen Gene of *Rickettsia tsutsugamushi*: Sequence Homology and Antigenic Comparison of Sta58 to the 60-Kilodalton Family of Stress Proteins

CHARLES K. STOVER,^{1†} DAVID P. MARANA,^{1‡} GREGORY A. DASCH,² AND EDWIN V. OAKS^{1*}

Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20307,¹ and
Division of Rickettsial Diseases, Naval Medical Research Institute, Bethesda, Maryland 20814²

Received 14 November 1989/Accepted 18 January 1990

The scrub typhus 58-kilodalton (kDa) antigen (Sta58) of *Rickettsia tsutsugamushi* is a major protein antigen often recognized by humans infected with scrub typhus rickettsiae. A 2.9-kilobase *Hind*III fragment containing a complete *sta58* gene was cloned in *Escherichia coli* and found to express the entire Sta58 antigen and a smaller protein with an apparent molecular mass of 11 kDa (Stp11). DNA sequence analysis of the 2.9-kilobase *Hind*III fragment revealed two adjacent open reading frames encoding proteins of 11 (Stp11) and 60 (Sta58) kDa. Comparisons of deduced amino acid sequences disclosed a high degree of homology between the *R. tsutsugamushi* proteins Stp11 and Sta58 and the *E. coli* proteins GroES and GroEL, respectively, and the family of primordial heat shock proteins designated Hsp10 Hsp60. Although the sequence homology between the Sta58 antigen and the Hsp60 protein family is striking, the Sta58 protein appeared to be antigenically distinct among a sample of other bacterial Hsp60 homologs, including the typhus group of rickettsiae. The antigenic uniqueness of the Sta58 antigen indicates that this protein may be a potentially protective antigen and a useful diagnostic reagent for scrub typhus fever.

Rickettsia tsutsugamushi, the causative agent of the sometimes fatal febrile illness known as scrub typhus, is an obligate intracellular bacterium that is transmitted to humans by the mite vector *Leptotrombidium deliense* (52). In humans, immunity after infection lasts for 1 to 3 years against the homologous strain but is short lived (less than 1 year) against heterologous strains (46, 47). Studies with laboratory animals have shown that both cellular and humoral immune responses occur after infection, with the cellular immune response being required for protection (16, 19, 25, 42). Humans infected with scrub typhus rickettsiae produce serum antibodies to at least eight rickettsial proteins, including the 110-, 58-, 56-, and 47-kilodalton (kDa) polypeptides (Karp strain; E. V. Oaks, unpublished data). Of these scrub typhus antigens (Sta), the 56-kDa major outer membrane protein (Sta56) and the 58-kDa protein (Sta58) are among the most abundant proteins in this rickettsia and are the antigens most often recognized by infected animals and humans (13, 51, 53; E. V. Oaks, unpublished data). At least two antigens (Sta110 and Sta56) have strain-variable molecular weights, contain strain-specific epitopes, and are possibly involved in the strain-specific protective immune response (10, 13, 32, 51). The Sta58 antigen appears to be more highly conserved among scrub typhus rickettsiae, since all strains tested to date express indistinguishable epitopes on a polypeptide that consistently runs at an apparent molecular mass of 58 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32).

Because sufficient quantities of this intracellular pathogen

are difficult to obtain, the genes encoding all the major antigens and some of the minor antigens of *R. tsutsugamushi* have been cloned and expressed in *Escherichia coli* to facilitate the evaluation of these potentially protective antigens as components of a subunit vaccine and future diagnostic procedures (32, 33, 50). In this paper, we report the molecular cloning and expression of the gene encoding the Sta58 protein antigen in *E. coli*. DNA sequence analysis of a 2.9-kilobase (kb) *Hind*III fragment carrying the Sta58 gene revealed two adjacent open reading frames (ORFs) potentially encoding scrub typhus proteins of 11 and 60 kDa (*stp11* and *sta58*, respectively). The deduced amino acid sequence of the *sta58* ORF was found to be homologous to those of the bacterial common antigens and the highly conserved family of 60-kDa heat shock proteins known collectively as Hsp60 stress proteins (15, 27, 41, 48, 56, 58).

The Hsp60 homologs from a number of microbial pathogens are being evaluated as potential vaccine components, in part because they are abundant proteins and are usually recognized by the immune system of the infected host (39, 55, 56). Similarly, the Sta58 protein is one of the most abundant proteins in *R. tsutsugamushi* that is also immunogenic. The Hsp60 protein family exhibits a high degree of homology and antigenic cross-reactivity, with very few unique determinants having been described (15, 41, 48, 56). The high degree of conservation of Hsp60 protein subunits could potentially trigger an autoimmune response to the human Hsp60 homolog (54). In this study, we demonstrate that a substantial degree of heterogeneity is also possible within the highly homologous framework of the Hsp60 family, as only weak serological cross-reactivity was observed between the Sta58 antigen and the Hsp60 homologs of some other common bacterial pathogens. A surprising lack of antigenic similarity between the Sta58 protein and the Hsp60 homolog of *Rickettsia typhi* may also indicate that *R.*

* Corresponding author.

† Present address: Molecular Vaccines Incorporated, 19 Firstfield Road, Gaithersburg, MD 20878.

‡ Present address: Division of Rickettsial Diseases, Naval Medical Research Institute, Bethesda, MD.

tsutsugamushi is more distantly related to the other members of the genus *Rickettsia* than was previously thought.

MATERIALS AND METHODS

Bacterial strains, media, and passage and preparation of *R. tsutsugamushi*. A plaque-purified Karp strain of *R. tsutsugamushi* was used throughout this study. The passage history of this strain was embryonated chicken eggs (52 times), L cells (3 times), and embryonated chicken eggs (6 times). Rickettsiae were grown in mouse fibroblasts (L-929 cells) incubated at 34°C in a humidified atmosphere of 5% CO₂-95% air. The rickettsiae were harvested 5 to 7 days postinfection and subsequently purified on Renografin (Squibb Diagnostics, New Brunswick, N.J.) density gradients as previously described (33). *E. coli* host strains and vectors are described below. All *E. coli* strains were grown in Luria broth or Luria broth agar (24). Luria broth was supplemented with ampicillin (100 µg/ml) for *E. coli* containing all plasmid pBR322 derivatives.

Recombinant DNA procedures. *R. tsutsugamushi* genomic DNA was isolated from purified rickettsiae as previously described (33). All restriction enzymes, T4 DNA ligase, and bacterial alkaline phosphatase were obtained from New England Biolabs (Beverly, Mass.) and used per the manufacturer's instructions. λgt11 libraries were constructed in *E. coli* host strain Y1090 (57) by using a modified method with conversion adaptor oligonucleotides as described by Stover et al. (50). Antigen-positive recombinant bacteriophage were identified by screening with hyperimmune rabbit sera (33, 50). Two recombinant phages (λgt11Rts224 and λgt11Rts408) (32) expressing Sta58 epitopes were used as a source of DNA for probing plasmid libraries. Recombinant λgt11 phage DNA was purified as described by Silhavy et al. (44). Cloned inserts were separated from recombinant λgt11 phage DNA by *Eco*RI digestion and agarose gel electrophoresis on 0.5% low-melting-point agarose (International Biotechnologies, Inc., New Haven, Conn.). Restriction fragment bands were excised, melted at 60°C in 50 mM Tris (pH 8.0)-100 mM NaCl buffer, and purified by using Elu-Tips (Schleicher & Schuell, Inc., Keene, N.H.). Plasmid DNA was prepared by a modification (26) of the procedure described by Birnboim and Doly (3). Plasmid DNA was transformed into *E. coli* HB101 competent cells by using the manufacturer's specifications (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). DNA probes were labeled by nick translation with the Bethesda Research Laboratories nick translation kit and [α-³²P]dCTP (Dupont, NEN Research Products, Boston, Mass.). All agarose (FMC Bioproducts, Rockland, Maine) gel electrophoresis was performed in Tris-acetate buffer, and DNA was visualized with ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) as described by Maniatis et al. (26). Southern blot hybridization and colony blot in situ hybridization were performed as described by Maniatis et al. (26).

Overlapping restriction fragments of plasmid pRTS58H2.9 were cloned into the replicative forms of M13mp18 and M13mp19 vectors (29, 37). XL1-Blue cells were transformed with the recombinant M13 replicative-form DNA (Stratagene, La Jolla, Calif.) and plated on strain TG1 (Amersham Corp., Piscataway, N.J.). Double- and single-stranded DNAs from transfected cultures were prepared as described by Ausubel et al. (1). The complete DNA sequences of both strands of the 2.9-kb *Hind*III restriction fragment of plasmid pRTS58H2.9 were determined by using the modified Sanger dideoxy-chain termination method (38) and [³⁵S]dATP (Du-

pont, NEN) essentially as described by Johnston-Dow et al., using the Sequenase kit (United States Biochemical, Cleveland, Ohio) (18). Oligonucleotide primers were synthesized on a Milligen Biosearch 8700 by using phosphoramidite chemistry (2). Sequencing-gel electrophoresis was performed on 6% polyacrylamide-7 M urea denaturing gels (Bio-Rad Laboratories, Rockville Centre, N.Y.). Dried gels were autoradiographed by using SB or BB (Eastman Kodak Co., Rochester, N.Y.) X-ray film. Assembly and analysis of the DNA sequence and derived protein sequences were accomplished with the University of Wisconsin Genetic Computer Group Sequence Analysis Software Package (9).

Identification of plasmid-encoded proteins. Plasmid-encoded proteins were identified in *E. coli* HB101 by using a modified maxicell procedure (36, 49). Modifications were as follows: HB101 was used instead of CSR603; the medium volume was doubled with M9 containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) and ampicillin (100 µg/ml) immediately after UV irradiation; and freshly prepared cycloserine (200 µg/ml; Sigma) was added 2 h after irradiation and 2 h before harvesting and labeling of the irradiated HB101 recombinant cells with [³⁵S]methionine (Dupont, NEN). Labeling was carried out in M9-1.2% methionine assay medium (Difco). The labeled maxicell preparations were analyzed by discontinuous 13% SDS-polyacrylamide gel electrophoresis (31) with the buffers described by Laemmli (23). Autoradiography using Kodak Blue Brand X-ray film was performed on the separated proteins after they were electroblotted onto nitrocellulose.

Protein electrophoresis and Western blotting. Rabbit anti-*R. tsutsugamushi* antiserum was prepared from a rabbit inoculated with gradient-purified whole *R. tsutsugamushi* Karp, which was also used for Western blot (immunoblot) analysis. This serum was exhaustively absorbed with *E. coli* for use in Western blot analysis of recombinant *E. coli* as described previously (33). Anti-BCG serum was raised in BALB/c mice to a cell wall fraction prepared from *Mycobacterium bovis* BCG (bacillus Calmette-Guérin) (Ribi Immunochem Research, Inc., Hamilton, Mont.). Polyvalent antiserum to the *Coxiella burnetii* HtpB protein (Hsp60 homolog) was raised to a peptidoglycan fraction from a recombinant *E. coli* strain expressing the *C. burnetii* HtpB antigen as described by Vodkin and Williams (55). The Nine Mile strain of *C. burnetii* was grown and purified as described by Vodkin and Williams (55). The BCG cell wall fraction, purified *C. burnetii* Nine Mile, and antisera to the BCG cell wall fraction and *C. burnetii* HtpB were kindly provided by Jim C. Williams, U.S. Army Research Institute of Infectious Diseases, Frederick, Md. SDS-PAGE and Western blotting of rickettsial polypeptides were performed as previously described (4, 23, 31). Staphylococcal protein A conjugated with alkaline phosphatase (Cappel, Organon Teknika Corp., West Chester, Pa.) was used to detect the antibody bound to antigens in the Western blot assay. Alkaline phosphatase-conjugated probes were developed with fast red TR salt and naphthol AS-MX phosphate as previously described (43).

RESULTS

Molecular cloning of a complete *sta58* gene. Fragments of the gene encoding the 58-kDa protein antigen of *R. tsutsugamushi* had been previously cloned in λgt11 (32, 50). Purified restriction fragment inserts from λgt11 clones (clones λgt11Rts224 and λgt11Rts408) (32) expressing antigenic determinants of the Sta58 protein were used as nucleic

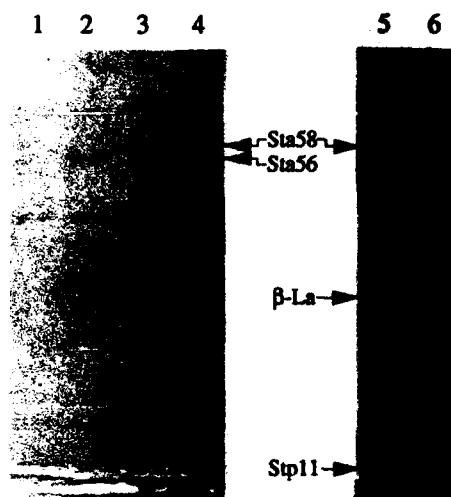


FIG. 1. Western blot and maxicell analysis of plasmid pRTS58H2.9-encoded proteins. The left panel is a Western blot reacted with rabbit anti-*R. tsutsugamushi* (strain Karp) against HB101(pBR322) (lane 1), HB101(pRTS56H2.3) (lane 2), HB101(pRTS58H2.9) (lane 3), and *R. tsutsugamushi* Karp (lane 4). The Sta56 antigen expressed in either *E. coli* or *R. tsutsugamushi* is indicated in lanes 2 and 4 of the Western blot, respectively. The recombinant and rickettsial Sta58 antigens of *R. tsutsugamushi* are indicated in lanes 3 and 4, respectively. To the right is an autoradiograph of [³⁵S]methionine-labeled maxicell preparations of HB101(pBR322) (lane 5) and HB101(pRTS58H2.9) (lane 6) separated by SDS-PAGE. The Sta58 antigen and Stp11 protein, both encoded by pRTS58H2.9, are indicated in lane 6 of the maxicell SDS-PAGE autoradiograph. Stp11 was not recognized by the serum used in the Western blot (lane 3). The β -lactamase protein encoded by the pBR322 vector (β -La) is indicated in lanes 5 and 6 of the maxicell preparations.

acid probes to identify restriction fragments associated with the *sta58* gene by Southern blot hybridization (data not shown). A 2.9-kb *Hind*III fragment, identified in this manner, was cloned into the *Hind*III site of pBR322 and identified by colony blot in situ hybridization with the *sta58* nucleic acid probes. The resulting plasmid, designated pRTS58H2.9, was found to express proteins with apparent molecular masses of 11 and 58 kDa by SDS-PAGE maxicell analysis (Fig. 1). Western blot analysis showed that the recombinant 58-kDa protein reacted with rabbit hyperimmune anti-*R. tsutsugamushi* serum that had been preabsorbed with *E. coli*. The recombinant protein comigrated with the 58-kDa antigen of *R. tsutsugamushi* (Fig. 1, lanes 3 and 4). The small apparent molecular mass difference between the recombinant Sta58 antigen and the Sta56 strain-specific major antigen of *R. tsutsugamushi* (strain Karp) could also be demonstrated (Fig. 1, lanes 2 and 4 [recombinant and native Sta56, respectively]). The complete recombinant 58-kDa protein also reacted with anti-Sta58 serum that had been affinity purified by using recombinant antigen from λ gt11 clones expressing smaller antigenic fragments of the Sta58 antigen (data not shown). The smaller 11-kDa recombinant protein did not react with the rabbit anti-*R. tsutsugamushi* serum by Western blot analysis.

Sequence analysis of the DNA encoding Stp11 and Sta58. The 2.9-kb *Hind*III fragment carrying the gene for the Sta58 protein was subjected to DNA sequence analysis (Fig. 2). The 33% G+C content of the sequenced 2.9-kb *Hind*III restriction fragment is comparable with the *R. tsutsugamushi* genomic G+C content of 35% (C. K. Stover, unpub-

lished data). Translations of all possible reading frames revealed two ORFs separated by 72 bases (Fig. 2). These two ORFs, designated *stp11* and *sta58*, were only marginally less AT-rich (36% G+C) than the entire 2.9-kb *Hind*III fragment. The 11- and 60-kDa coding capacities of these two ORFs were in reasonable agreement with the sizes of the recombinant 11- and 58-kDa gene products identified by maxicell analysis. Polypurine-rich potential ribosomal binding sites were located 4 bases from the start codon of the *stp11* ORF and 8 bases from the start codon of the *sta58* ORF. Reasonable matches to the *E. coli* promoter -10 and -35 consensus sequences were also found 110 bases 5' from the likely start codon of the *sta11* gene (Fig. 2).

Homology searches using the derived amino acid sequence encoded by the *sta58* ORF disclosed striking identity to the *E. coli* GroEL protein, the mycobacterial 65-kDa antigens, the *C. burnetii* HtpB protein, and the large family of 60 kDa stress proteins (data not shown) (15, 41, 48, 56, 58). Figure 3 depicts a best-fit amino acid sequence alignment of the *R. tsutsugamushi* Sta58 protein and the published sequences of other Hsp60 proteins from four bacterial genera, one plant, one yeast, and humans (14, 17, 28, 35, 55). The amino acid sequence homology between the deduced Sta58 protein and the Hsp60 family averaged 49% identical residues (Fig. 3). Alignment of hydrophobicity curves for each of the seven Hsp60 homologs disclosed superimposable hydrophobic profiles, indicating that a substantial percentage of the nonidentical amino acids are of conserved type (Fig. 4). Further analysis revealed that approximately 40% of the nonidentical amino acids are conservative replacements, yielding an average sequence similarity of approximately 70%. The alignment of the seven Hsp60 sequences revealed at least four hyperconserved regions along the Hsp60 polypeptide backbone. The C-terminal region of the Sta58 sequence contained the same repetitive Gly-Gly-Met motif observed in all Hsp60 homologs except the plant *Triticum aestivum* (wheat) (14). It has been suggested that the hydrophobic C-terminal region of this protein family is transmembranous and that the hydrophilic repetitive Gly-Gly-Met sequence at the extreme C terminus may serve to anchor this protein.

Previous sequence analysis of regions encoding the Hsp60 homologs of *E. coli* (GroEL), *C. burnetii* (HtpB), and *Synechococcus* strain 6301 (Urf4) had also identified a gene encoding a 10- to 11-kDa protein 5' to, and probably cotranscribed with, the *hsp60* gene (7, 14, 55). These smaller 10- to 12-kDa polypeptides are designated Hsp10 proteins. Computer-assisted best-fit alignments between the derived amino acid sequence of the recombinant *R. tsutsugamushi* Stp11 protein and the derived amino acid sequences of *E. coli* GroES, *C. burnetii* HtpA, *Mycobacterium tuberculosis* 12-kDa antigen, and *Synechococcus* strain 6301 Urf3 disclosed an average sequence identity of 46%. As in the comparisons of Hsp60 sequences, a substantial percentage of the nonidentical amino acids were similar, resulting in generally superimposable hydrophobic profiles (Fig. 4). Homology between four bacterial GroES homologs was found to be uniform throughout the length of the relatively small polypeptide backbone.

Other studies have shown that the *E. coli* stress protein GroEL and the Hsp60 homologs of *C. burnetii* (HtpB), *M. tuberculosis*, and *Mycobacterium smegmatis* (65-kDa antigen) are heat shock proteins (29, 41, 55). Potential $E. coli$ heat shock promoter -10 and -35 regions have been found 5' to the *groES-groEL* gene operon of *E. coli* and the *htpA-htpB* operon of *C. burnetii* (6, 14, 55). Expression of the recom-

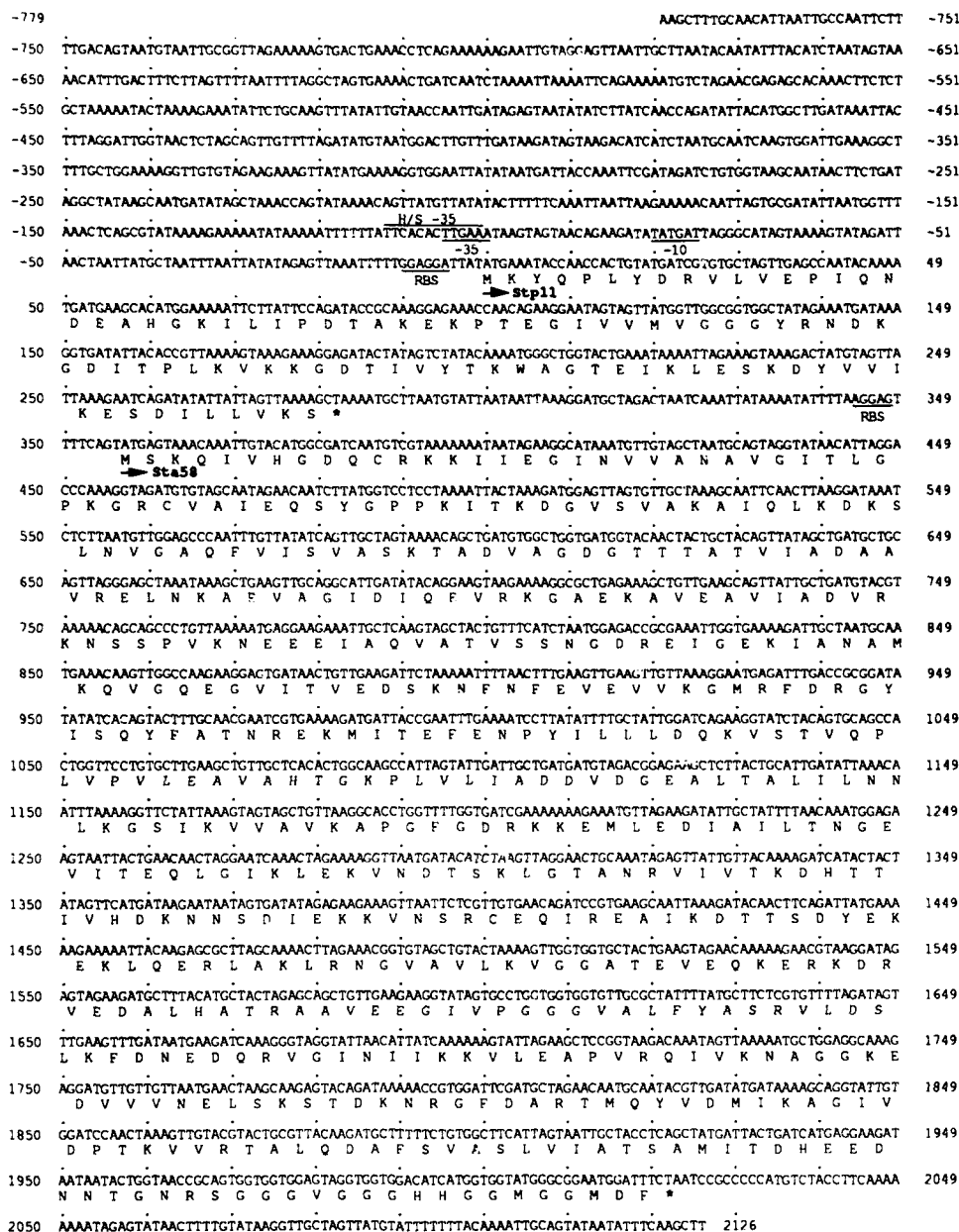


FIG. 2. Nucleotide sequence and deduced amino acid sequences of the *stp11*-*sta58* genes and flanking 5' and 3' regions. ORFs encoding the *Stp11* and *Sta58* proteins are denoted by rightward-pointing (5' to 3') arrows. The first base of the presumed *Stp11* initiation codon is numbered 0. Sequences resembling the consensus sequences for ribosomal binding sites (RBS) and promoter -10 and -35 regions are underlined. A 12-base-pair sequence resembling the consensus sequence for a heat shock promoter -35 region (H/S -35) is overlined. Asterisks denote stop codons. GenBank accession number is M31887.

binant *Sta58* stress protein homologs did not appear to be stimulated at higher temperatures (44°C) in *E. coli* (data not shown). However, a good match to the *Eo*³² heat shock promoter -35 region was found overlapping the putative *stp11*-*sta58* *Eo*⁷⁰ -35 region proposed above (Fig. 2). A potential *Eo*³² heat shock promoter -10 region was not identified.

Antigenic uniqueness of *Sta58* among the family of Hsp60 stress proteins. The first evidence of a 60-kDa common antigen family (Hsp60) among procaryotes was based on serum cross-reactivity (15). Antigenic cross-reactivity between the *R. tsutsugamushi* *Sta58* and the Hsp60 common

antigens has not been previously reported. Because of the strong sequence homology observed between the *R. tsutsugamushi* *Sta58* protein and the Hsp60 family of stress proteins, the *Sta58* antigen was compared with other bacterial Hsp60 homologs by Western blot analysis with polyvalent and monoclonal antisera known to react with bacterial Hsp60 antigens (Fig. 5). Polyclonal antiserum to a BCG cell wall fraction and the *C. burnetii* Hsp60 homolog (62-kDa HtpB antigen) reacted strongly with protein antigens in the 60-kDa range for all the bacteria tested except that of *R. tsutsugamushi*. The same pattern of strong reactivity seen with the polyvalent BCG and *C. burnetii* antisera was

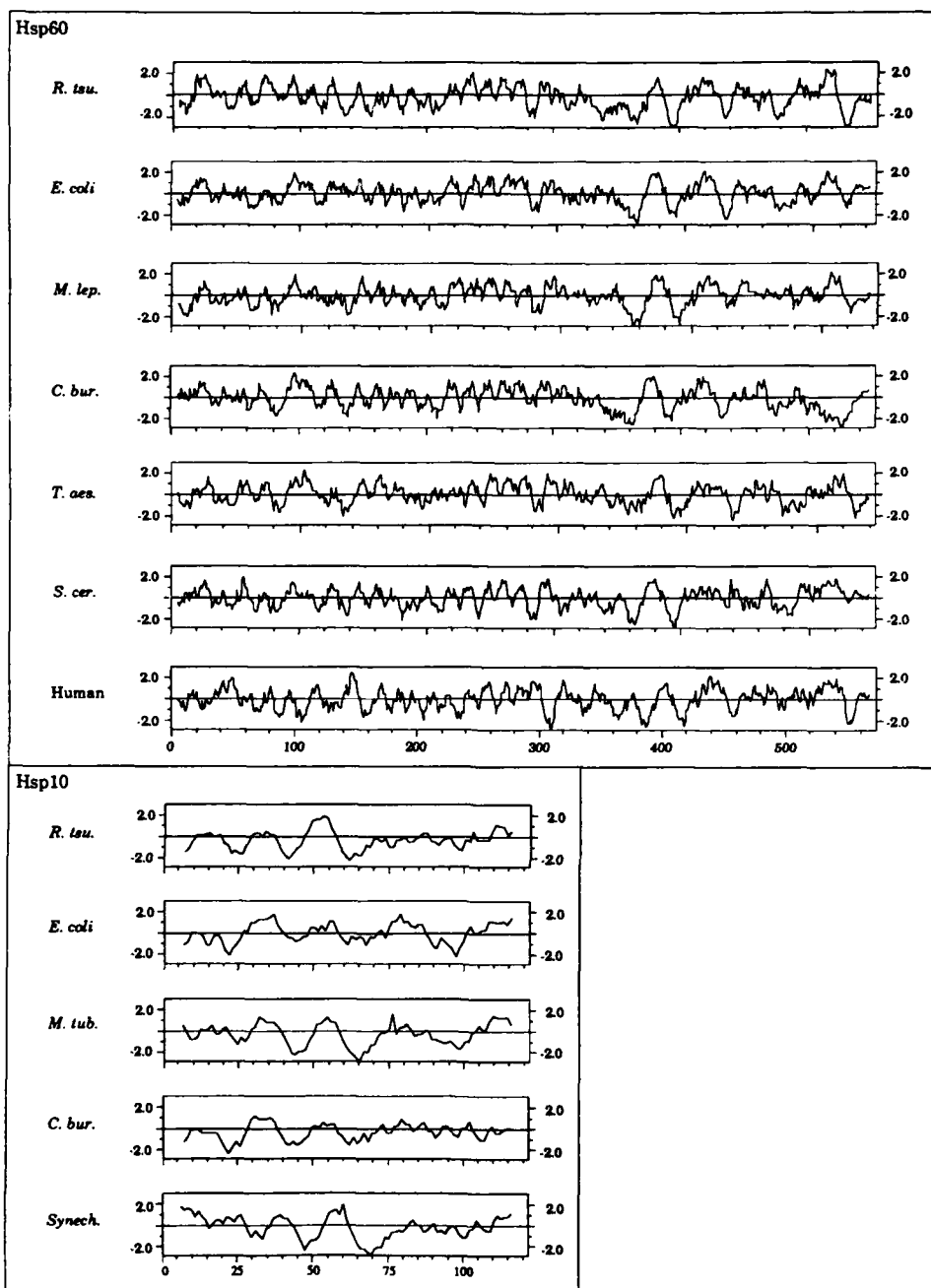


FIG. 4. Comparison of Hsp10 and Hsp60 hydrophobicity profiles. Hydrophobicity was plotted by the method of Kyte and Doolittle (22). Regions above the horizontal axis are hydrophobic. Abbreviations are defined in the legend to Fig. 3.

expression of the *R. tsutsugamushi* *sta58* gene in *E. coli* will facilitate further evaluation of the Sta58 protein as a possible component of future scrub typhus subunit vaccines. On the basis of overwhelming amino acid sequence identity (average, 49%) and similarity we conclude that the Sta58 protein antigen is a member of the Hsp60 family of stress proteins, also termed common antigens. However, among the complete bacterial Hsp60 sequences known, the *R. tsutsugamushi* Sta58 antigen appears to be the least similar. In contrast to the observed genetic relatedness of Sta58 and Hsp60 proteins, Western blot analysis of a number of bacteria with a variety of polyclonal and monoclonal antisera (known to react with Hsp60 proteins) indicated that the *R.*

tsutsugamushi Sta58 protein is also unique antigenically. Although observations, including distinct ultrastructure and unique physiological requirements, have suggested that *R. tsutsugamushi* is clearly unique among other members of the genus *Rickettsia* (21, 45), it was unexpected that we would find a lack of antigenic similarity between the *R. tsutsugamushi* Sta58 antigen and the Hsp60 homolog of *R. prowazekii*, a member of the typhus group of rickettsiae. This result is particularly surprising because substantial antigenic similarity was observed between *Rickettsia prowazekii* and the Hsp60 homologs of other, presumably less-related bacteria. It should be noted that this study used a set of five monoclonal antibodies (including those shown in

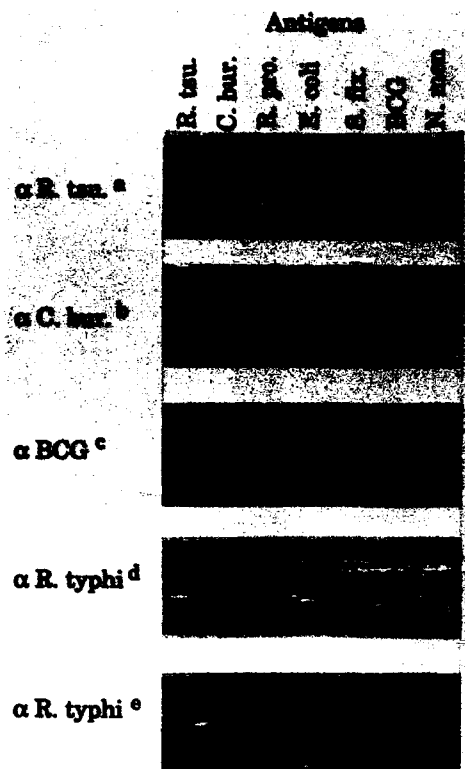


FIG. 5. Serological comparison of Hsp60 common antigen homologs by Western blot analysis. Various antigen preparations (*R. tsu.*, *R. tsutsugamushi*; *C. bur.*, *C. burnetii*; *R. pro.*, *R. prowazekii*; *S. flex.*, *Shigella flexneri*; BCG, *M. bovis* BCG; *N. men.*, *Neisseria meningitidis*) were electrophoresed and electroeluted onto nitrocellulose. Each panel represents a similar gel with each battery of antigens. Only the portion of the gel containing the Hsp60 antigens is shown. Each antigen group was reacted separately with the antisera designated on the left (a, polyclonal antiserum to gradient-purified *R. tsutsugamushi* Karp; b, polyclonal antiserum to recombinant *C. burnetii* HtpB protein [55]; c, polyclonal antiserum to BCG cell wall fraction; d, monoclonal antiserum TC30-2F2.2 to purified *R. typhi* Hsp60; e, monoclonal antiserum T5-1F1.5 to purified *R. typhi* Hsp60).

Fig. 5) specific for five unique epitopes of the *R. typhi* Hsp60 antigen which clearly react with *R. prowazekii* (G. A. Dasch, manuscript in preparation). The Western blot data do not preclude the possibility that the *R. tsutsugamushi* Sta58 protein shares common antigenic determinants with Hsp60 homologs of other bacteria. In fact, some marginal cross-reactivity was observed between the Sta58 protein and the *C. burnetii* HtpB antigen. From this limited survey one may conclude that the *R. tsutsugamushi* Sta58 antigen shares fewer common epitopes with the Hsp60 family than the Hsp60 proteins of the other bacteria used in this study. These data also suggest that *R. tsutsugamushi* may be more distantly related to the other rickettsiae than was previously thought. Molecular cloning and sequence analysis of the spotted-fever and typhus group *Rickettsia* hsp60 genes coupled with more detailed serological studies of the *Rickettsia* Hsp60 homologs may give interesting insights into the taxonomic relationship between *R. tsutsugamushi* and the other *Rickettsia* groups. For example, it was surprising to find that the *C. burnetii* HtpB Hsp60 homolog is substantially closer in identity to the *E. coli* GroEL protein than to the Sta58

protein, even though the genus *Coxiella* is in the tribe *Rickettsieae* and was once in the genus *Rickettsia* (8).

Because it is believed that the Hsp10 proteins and Hsp60 proteins complex with each other, it is possible that the *stp11* and *sta58* genes are cotranscribed or coregulated (14). Before this study, the genes encoding Hsp10 and Hsp60 protein homologs had been found to be closely linked, and probably cotranscribed, from *hsp10* to *hsp60* in *E. coli*, *C. burnetii*, and *Synechococcus* strain 6301 (14, 55). In this study, the location of promoterlike sequences 5' to the *stp11* gene, the apparent absence of promoterlike sequences for the adjacent downstream *sta58* gene, and the lack of recognizable transcriptional terminators between the two genes suggests that the *stp11* and *sta58* genes may be cotranscribed in the same order as the *hsp10* and *hsp60* genes of *E. coli*, *C. burnetii*, and *Synechococcus* strain 6301. Exceptions to the close gene linkage include the *hsp10* (BCG-a gene) and *hsp60* genes of *M. tuberculosis* (40) and genes encoding Hsp60 homologs from a number of plants and other eucaryotes, including humans (5, 14, 17).

One of the eventual goals of this effort is to evaluate the *R. tsutsugamushi* Sta58 major protein antigen as a potentially protective antigen. Because the Hsp60 proteins are generally immunodominant antigens, a number of groups are also studying the Hsp60 proteins of other procaryotic and eucaryotic pathogens as protective antigens (39, 55, 56). A major concern regarding the use of an Hsp60 antigen or any other highly conserved protein in a vaccine is the potential for the antigen to elicit an autoimmune response to a homologous self protein. A number of bacterial pathogens have been associated with autoimmune disorders (including arthritis). In the case of the rat adjuvant arthritis model, arthritogenic T cells have been shown to cross-react with the *M. tuberculosis* 65-kDa antigen and an unidentified protein in synovial fluid, but it remains to be determined whether this cross-reactivity is due to a specific immune response to the Hsp60 of the pathogen. Recent evidence has prompted the hypothesis that the immune system is actually predisposed to autoreact with self Hsp60 (and possibly other highly conserved stress proteins) and that this autoimmune response is central to a normal mechanism for immune surveillance (20, 30, 34; for a review, see reference 58).

Alternatively, it has been questioned whether the highly homologous stress proteins could have any protective vaccine potential at all, since infection by one pathogen does not normally protect an individual from infection with a different pathogen. It has been suggested by Young et al. (56) that exposure to bacterial stress protein families early in life may induce immunity to a variety of different pathogens and could explain why only a fraction of individuals infected with some pathogens actually acquire clinical disease. It is our belief that further study of the *R. tsutsugamushi* Sta58 antigen and Hsp60 proteins as protective antigens is warranted because it is clear from previous studies and this study that within the highly homologous framework of the Hsp60 proteins there is a considerable capacity for unique antigenic determinants and, therefore, the possibility for specific protective immunity. Because lasting protection against scrub typhus rickettsiae is strain specific, the Sta58 antigen by itself may not be sufficient for a protective subunit vaccine, as no apparent strain-specific differences in the *R. tsutsugamushi* Sta58 antigen have been observed to date (32). Nevertheless, initial short-term heterologous protection may indicate that an antigen or antigens common to the strains of *R. tsutsugamushi* might provide marginal short-term protection. It is also possible that marginally protective

antigen components are necessary for a complete and effective vaccine.

In addition to their potential as components in protective vaccines, recombinant Hsp60 homologs from a variety of organisms may be useful for the stability and correct assembly of recombinant foreign proteins. While the Hsp60 proteins probably carry out similar functions in their respective hosts, there are some indications that they are not completely interchangeable and that Hsp60 proteins may have some preference for proteins of their normal environment. For example, a temperature-sensitive *E. coli* GroEL mutant could not be suppressed by a recombinant *M. tuberculosis* homolog (41). It has also been demonstrated that hyperexpression of a nonnative Hsp10-Hsp60 can permit the correct assembly of an enzyme complex that normally requires the assembly of the Hsp10-Hsp60 complex in its normal host (e.g., plant ribulose 1,5-bisphosphate carboxylase-oxygenase) (11). It is plausible that the problem of incorrect assembly of recombinant proteins may be circumvented by cloning the normal *hsp10* *hsp60* homologs in consort with the genes of interest. This possibility is made more tenable by the general finding that the Hsp60 homologs cloned so far appear to be expressed well and are stable in *E. coli*. The stable nature and possible intrinsic antigenicity of the Hsp10-Hsp60 homologs might also make this family of proteins useful as protein carriers for different antigens to be used in vaccine preparations. It is therefore conceivable that the recombinant *stp11-sta58* genes could be used not only as a vaccine immunogen but also to assist in the stable expression and presentation of other recombinant *R. tsutsugamushi* antigens.

(It should be noted that the genes encoding the cyanobacterium *Synechococcus* strain 6301 Hsp10 and Hsp60 proteins were not recognized as such but were fortuitously cloned and sequenced along with ATP synthase subunit genes [6]. It was later determined by sequence homology that two ORFs designated Urf4 and Urf4 probably encode the *Synechococcus* strain 6301 Hsp10 and Hsp60 homologs [M. Vodkin, personal communication]. The Urf4 (Hsp60 homolog) ORF is not completely sequenced and is truncated at about 900 bases [300 amino acids].)

ACKNOWLEDGMENTS

We thank Mike Vodkin, Jerry Buysse, and Bruce Roe for their valuable advice and discussion. We also thank Malabi Venkatesan for oligonucleotide synthesis support, Elaine Mardis and Stephanie Pyle for their technical assistance, and Chris Bolt, Tim Hoover, and Jim Williams for the *Coxiella* and BCG antisera and antigens.

LITERATURE CITED

- Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
- Beaucage, S. L., and M. H. Caruthers. 1981. Deoxynucleotide phosphoramides—a new class of key intermediates for deoxy-polynucleotide synthesis. *Tetrahedron Lett.* 22:1859–1862.
- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513–1523.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
- Cheng, M., F.-U. Hart, J. Martin, R. Pollock, F. Kalousek, W. Neupert, E. Hallberg, R. Hallberg, and A. Horwich. 1989. Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature (London)* 337:620–625.
- Cowing, D. C., J. C. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix, and C. A. Gross. 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. *Proc. Natl. Acad. Sci. USA* 82:2679–2683.
- Cozens, A., and J. Walker. 1987. The organization and sequence of the genes for ATP synthase subunits in the cyanobacterium *Synechococcus* 6301. *J. Mol. Biol.* 194:359–383.
- Derrick, E. A. 1939. *Rickettsia burnetii*: the cause of "Q" fever. *Med. J. Aust.* 1:14.
- Deveraux, J., P. Haeblerli, and O. Smithies. 1984. University of Wisconsin genetic computer group sequence analysis software package. *Nucleic Acids Res.* 12:387–395.
- Eisemann, C. S., and J. V. Osterman. 1985. Identification of strain-specific and group reactive antigenic determinants on the Karp, Gilliam, and Kato strains of *Rickettsia tsutsugamushi*. *Am. J. Trop. Med. Hyg.* 34:1173–1178.
- Goloubinoff, P., A. Gatenby, and G. Lorimer. 1989. GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose biphosphate carboxylase oligomers in *Escherichia coli*. *Nature (London)* 337:44–47.
- Hanson, B. 1984. Characterization of *Rickettsia tsutsugamushi* outer membrane proteins. p. 240–243. In L. Lieve and D. Schlessinger (ed.), *Microbiology—1984*. American Society for Microbiology, Washington, D.C.
- Hanson, B. 1985. Identification and partial characterization of *Rickettsia tsutsugamushi* major protein immunogens. *Infect. Immun.* 50:603–609.
- Hemingsen, S., C. Woolford, S. van der Vies, K. Tilly, D. Dennis, C. Georgopoulos, R. Hendrix, and R. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature (London)* 333:330–334.
- Hoiby, N. 1975. Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. *Scand. J. Immunol.* 4(Suppl. 2):187–196.
- Jerrells, T. R., B. A. Palmer, and J. G. MacMillan. 1984. Cellular mechanisms of innate and acquired immunity to *Rickettsia tsutsugamushi*, p. 277–281. In L. Lieve and D. Schlessinger (ed.), *Microbiology—1984*. American Society for Microbiology, Washington, D.C.
- Jindal, S., A. Dudani, B. Singh, C. Harley, and R. Gupta. 1989. Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. *Mol. Cell. Biol.* 9:2279–2283.
- Johnston-Dow, L., E. Mardis, C. Heiner, and B. A. Roe. 1987. Optimized methods for fluorescent and radiolabeled DNA sequencing. *BioTechniques* 5:754–765.
- Kodama, K., S. Kawamura, M. Yasukawa, and Y. Kobayashi. 1987. Establishment and characterization of a T-cell line specific for *Rickettsia tsutsugamushi*. *Infect. Immun.* 55:2490–2495.
- Koga, T., A. Wand-Wuittenberger, J. DeBruyn, M. Munk, B. Schoel, and S. Kaufmann. 1989. T-cells against a bacterial heat shock protein recognize stressed macrophages. *Science* 245:1112–1115.
- Kopmans-Gargantiel, A. I., and C. L. Wiseman, Jr. 1981. Differential requirements for enriched atmosphere carbon dioxide content for intracellular growth in cell culture among selected members of the genus *Rickettsia*. *Infect. Immun.* 31:1277–1280.
- Kyte, J., and F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680–685.
- Luria, S. E., and M. L. Human. 1952. A nonhereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* 64:557–569.
- Macmillan, J. G., R. M. Rice, and T. R. Jerrells. 1985. Development of antigen-specific cell-mediated immune responses after infection of *Cynomolgus* monkeys (*Macaca fascicularis*) with *Rickettsia tsutsugamushi*. *J. Infect. Dis.* 152:739–749.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular

- cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. McMullin, T. W., and R. Hallberg. 1988. A highly evolutionarily conserved protein is structurally related to the protein encoded by the *Escherichia coli* *groEL* gene. *Mol. Cell. Biol.* 8:371-380.
 28. Mehra, V., D. Sweetzer, and R. Young. 1986. Efficient mapping of protein antigenic determinants. *Proc. Natl. Acad. Sci. USA* 83:7013-7017.
 29. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* 101:20-78.
 30. Munk, M. E., B. Schoel, and S. Kaufmann. 1988. T cell responses of normal individuals towards recombinant protein antigens of *Mycobacterium tuberculosis*. *Eur. J. Immunol.* 18:1835-1838.
 31. Oaks, E. V., T. L. Hale, and S. B. Formal. 1986. Serum immune response to *Shigella* protein antigens in rhesus monkeys and humans infected with *Shigella* spp. *Infect. Immun.* 48:124-129.
 32. Oaks, E. V., D. J. Kelly, R. Rice, and C. K. Stover. 1989. Antigenic and genetic relatedness of eight *Rickettsia tsutsugamushi* antigens. *Infect. Immun.* 57:3116-3122.
 33. Oaks, E. V., R. M. Rice, and C. K. Stover. 1987. Molecular cloning and expression of *Rickettsia tsutsugamushi* genes for two major protein antigens in *Escherichia coli*. *Infect. Immun.* 55:1156-1157.
 34. O'Brien, R. L., M. P. Happ, A. Dallas, E. Palmer, R. Kubo, and W. K. Born. 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor $\gamma\delta$ by an antigen derived from *Mycobacterium tuberculosis*. *Cell* 57:667-674.
 35. Reading, D. S., R. Hallberg, and A. Myers. 1989. Characterization of the yeast *hsp60* gene coding for a mitochondrial assembly factor. *Nature (London)* 337:655-658.
 36. Sancar, A., A. M. Hack, and W. D. Rupp. 1979. Simple method for identification of plasmid-coded proteins. *J. Bacteriol.* 137:692-693.
 37. Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161-178.
 38. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
 39. Shinnick, T. M. 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J. Bacteriol.* 169:1080-1088.
 40. Shinnick, T. M., B. B. Plikaytis, A. D. Hyshe, R. M. Van Landingham, and L. L. Walker. 1989. The *Mycobacterium tuberculosis* BCG-a protein has homology with the *Escherichia coli* GroES protein. *Nucleic Acids Res.* 17:1254.
 41. Shinnick, T. M., M. Vodkin, and J. Williams. 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. *Infect. Immun.* 56:446-451.
 42. Shirai, A., P. J. Cantanzaro, S. M. Phillips, and J. V. Osterman. 1976. Host defenses in experimental scrub typhus: role of cellular immunity in heterologous protection. *Infect. Immun.* 14:39-46.
 43. Sidberry, H., B. Kaufman, D. C. Wright, and J. Sadoff. 1985. Immunoenzymatic analysis by monoclonal antibodies of bacterial lipopolysaccharides after transfer to nitrocellulose. *J. Immunol. Methods* 76:299-305.
 44. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 45. Silverman, D., and C. L. Wisseman, Jr. 1978. Comparative ultra-structural study on the cell envelopes of *Rickettsia prowazekii*, *Rickettsia rickettsii*, and *Rickettsia tsutsugamushi*. *Infect. Immun.* 21:1020-1023.
 46. Smadel, J. E., H. L. Ley, Jr., F. H. Diercks, P. Y. Paterson, C. L. Wisseman, Jr., and R. Traub. 1952. Immunization against scrub typhus: duration of immunity in volunteers following combined living vaccine and chemoprophylaxis. *Am. J. Trop. Med. Hyg.* 1:87-99.
 47. Smadel, J. E., H. L. Ley, F. H. Diercks, and R. Traub. 1950. Immunity in scrub typhus: resistance to induced reinfection. *Arch. Pathol.* 50:847-867.
 48. Sompolinsky, D., J. Hertz, N. Hoiby, K. Jensen, B. Mansa, and Z. Samra. 1980. An antigen common to a wide range of bacteria. I. A biochemical study of a common antigen from *Pseudomonas aeruginosa*. *Acta Pathol. Microbiol. Scand. Sect. B* 88:143-149.
 49. Stover, C. K., J. Kemper, and R. C. Marsh. 1988. Molecular cloning and characterization of *supQ/newD*, a gene substitution system for the *leuD* gene of *Salmonella typhimurium*. *J. Bacteriol.* 170:3115-3124.
 50. Stover, C. K., M. H. Vodkin, and E. V. Oaks. 1987. Use of conversion adaptors to clone antigen genes in λ gt11. *Anal. Biochem.* 163:398-407.
 51. Tamura, A., N. Ohashi, H. Urakami, K. Takahashi, and M. Oyanagi. 1985. Analysis of polypeptide composition and antigenic components of *Rickettsia tsutsugamushi* by polyacrylamide gel electrophoresis and immunoblotting. *Infect. Immun.* 48:671-675.
 52. Traub, R., and C. L. Wisseman, Jr. 1974. The ecology of chigger-borne rickettsiosis (scrub typhus). *J. Med. Entomol.* 11:237-303.
 53. Urakami, H., N. Ohashi, T. Takashi, and A. Tamura. 1986. Characterization of polypeptides in *Rickettsia tsutsugamushi*: effect of preparative conditions on migration of polypeptides in polyacrylamide gel electrophoresis. *Infect. Immun.* 51:948-952.
 54. van Eden, W., J. Thole, R. van der Zee, A. Noordzij, J. van Embden, E. Hensen, and I. Cohen. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature (London)* 331:171-173.
 55. Vodkin, M. H., and J. C. Williams. 1988. A heat shock operon in *Coxiella burnetii* produces a major antigen homologous to a protein in both mycobacteria and *Escherichia coli*. *J. Bacteriol.* 170:1227-1234.
 56. Young, D., R. Lathigra, R. Hendrix, D. Sweetser, and R. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc. Natl. Acad. Sci. USA* 85:4267-4270.
 57. Young, R. A., and R. W. Davis. 1983. Yeast RNA polymerase II genes: isolation with antibody probes. *Science* 222:778-782.
 58. Young, R. A., and T. J. Elliott. 1989. Stress proteins, infection, and immune surveillance. *Cell* 59:5-8.

Accession For	
NTIS	<input checked="" type="checkbox"/>
CRA&I	<input type="checkbox"/>
DTIC	<input type="checkbox"/>
TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution	
Availability Codes	
Dist	Avail and/or Special
A-1	20